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Short Communication

Expression and Localization of Tumor Necrosis Factor-α and Its mRNA in Idiopathic Pulmonary Fibrosis

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The expression of tumor necrosis factor α and its mRNA was investigated in surgical biopsies from idiopathic pulmonary fibrosis by immunohistochemistry, in situ hybridization, and Northern blotting. Normal areas of lungs resected for cancer were used as controls. Tumor necrosis factor α mRNA levels were higher in idiopathic pulmonary fibrosis than in normal lungs as determined by Northern blots. In normal lungs, tumor necrosis factor α and its mRNA were identified in alveolar and interstitial macrophages. In fibrotic lungs, tumor necrosis factor α was detected in macrophages and, to a greater extent, in epithelial cells (presumably type II cells) lining the thickened septae. Tumor necrosis factor α mRNA was found only in some interstitial cells and some of the cells lining the alveolar septae. An elevated concentration of tumor necrosis factor = α, particularly within the alveolar epithelium, might contribute to the alveolar damage and proliferation of interstitial cells in idiopathic pulmonary fibrosis. (Am J Pathol 1993, 143:651–655)

Idiopathic pulmonary fibrosis (IPF) is a progressive disorder characterized histologically by thickening of the alveolar septae by interstitial cells and inflammation.[1] It is generally assumed that the proliferation of interstitial cells is due to an overproduction of fibrogenic cytokines. Two, platelet-derived growth factor and transforming growth factor-β1, have been recently identified in foci of active IPF.[2,3] Tumor necrosis factor-α (TNF-α) is another cytokine with both inflammatory and fibrogenic properties.[4] It is possible that implication in IPF is suggested by investigations of murine lung fibrosis elicited by bleomycin or silica. In each model, there is a marked and lasting increase of TNF-α mRNA in the lung; moreover, this fibrosis can be prevented by the administration of anti–TNF-α antibody.[4]

The aim of this study was to investigate a possible role of TNF-α in the pathogenesis of IPF.

Materials and Methods

Biopsy Specimens

Fresh surgical pulmonary tissue from five patients with IPF was available for study; their case histories are summarized in Table 1. Six normal lung specimens, obtained from resection of cancer, served as controls. Tissues were snap frozen in liquid nitrogen and stored at −70 C. Frozen cryostat sections were prepared for immunohistochemical and *in situ* hybridization studies.

Antibodies and Immunohistochemistry

Anti–TNF-α antibodies: Mouse monoclonal (MoAb) anti-human TNF-α was obtained from Dr. W. Buurman (University of Limburg, 6200-MD Maastricht, The Netherlands, clone 52B83) and Dr. J. Wijdenes (Besançon, France, clone BC-7). Mouse mono-

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clonal anti-human keratin gpp 56 MoAb was purchased from Immunotech (F-78150, Le Chesnay, France). Mouse anti-monocyte/macrophage antibody (anti CD68 or KP1) was purchased from Dako (Glostrup, Denmark). Mouse IgGs were detected with a biotinylated horse anti-mouse IgG and the avidine-peroxidase complex (Vector Laboratories, Burlingame, CA).

Northern Blot Analysis of TNF-α

RNA was isolated by guanidine/cesium chloride centrifugation as described previously.[5] RNAs, denatured with glyoxal, were separated on 1.2% agarose gels (4 μg/lane) and transferred onto nylon membranes. Filters were hybridized with ^{32}P-labeled cRNA probes.[5] The quality of RNA and loading were evaluated by hybridization with a 1.1-kb chicken GAPDH cDNA, subcloned into a *Pst*I restriction site of pSp64.[6]

Detection of TNF-α mRNA by In Situ *Hybridization*

Human TNF-α cDNA probes[7] were transcribed *in vitro* in the presence of 100 μCi of ^{35}S UTP (Amersham, UK). *In situ* hybridization was performed as described elsewhere.[8]

Results

Normal Lung (TNF-α and its mRNA)

In six normal controls, as judged by conventional histology, anti–TNF-α antibodies weakly stained clusters of intra-alveolar cells and a few interstitial cells (Figure 1B and C). These cells were judged to be macrophages, because they stained strongly with anti-CD68 MoAb (Figure 1A). TNF-α mRNA was detected in rare interstitial and intra-alveolar cells by *in situ* hybridization (not shown). Neither the protein nor its mRNA was detected in alveolar epithelium.

IPF (TNF-α)

Alveoli appeared as irregular spaces, delimited by thickened septae (Figure 1G) containing numerous fibroblasts and myofibroblasts. Septae were lined by cuboidal layers of epithelial cells, which stained strongly with anti-keratin MoAb (Figure 1F) and were therefore in great numbers regenerating type II pneumocytes.

Staining with the two different anti–TNF-α MoAbs revealed intense labeling of cells lining the thickened septae (Figure 1D and H) in all five cases examined. This pattern contrasted with negative staining obtained with anti-CD+68 MoAb (not shown). A variable number of cells containing TNF-α was also detected in the interstitium (presumably macrophages) and in alveoli, although these were generally less intensively labeled than epithelial cells.

TNF-a mRNA Detected by In Situ *Hybridization*

A strong hybridization signal (ie, cells bearing a number of grains >5-fold above background) was obtained only in three of five cases examined in three separate hybridizations (Table 1). In these cases, a moderate accumulation of grains was detected on the cells within the alveoli (Figure 1I), and a more marked accumulation of grains in some rare interstitial cells and some of the cells lining the alveolar walls (Figure 1J). Failure to obtain significant hybridization in the remaining two cases was presumed to result either from a suboptimal handling of tissue specimens or possibly to immunosuppressive therapy, notably corticosteroids (Table 1), which might have affected transcription.[9]

Table 1. *Idiopathic Pulmonary Fibrosis*

n	Age and sex	Evolution and therapy	Histology	Results	
				TNF	TNF mRNA
1	42, M	3 years, no steroids	Advanced fibrosis	Epithelium and macrophages	Epithelium and macrophages
2	78, M	10 years, no steroids	Advanced fibrosis	Epithelium	Macrophages
3	47, F	5 months, steroids	Septal thickening, CD4+ lymphocyte and polymorphonuclear leukocyte	Epithelium and macrophages	Not detected
4	39, F	14 years, steroids	Advanced fibrosis	Epithelium and macrophages	Macrophages
5	62, F	2 years, no steroids	Septal thickening CD4+ lymphocytes	Epithelium and macrophages	Not detected

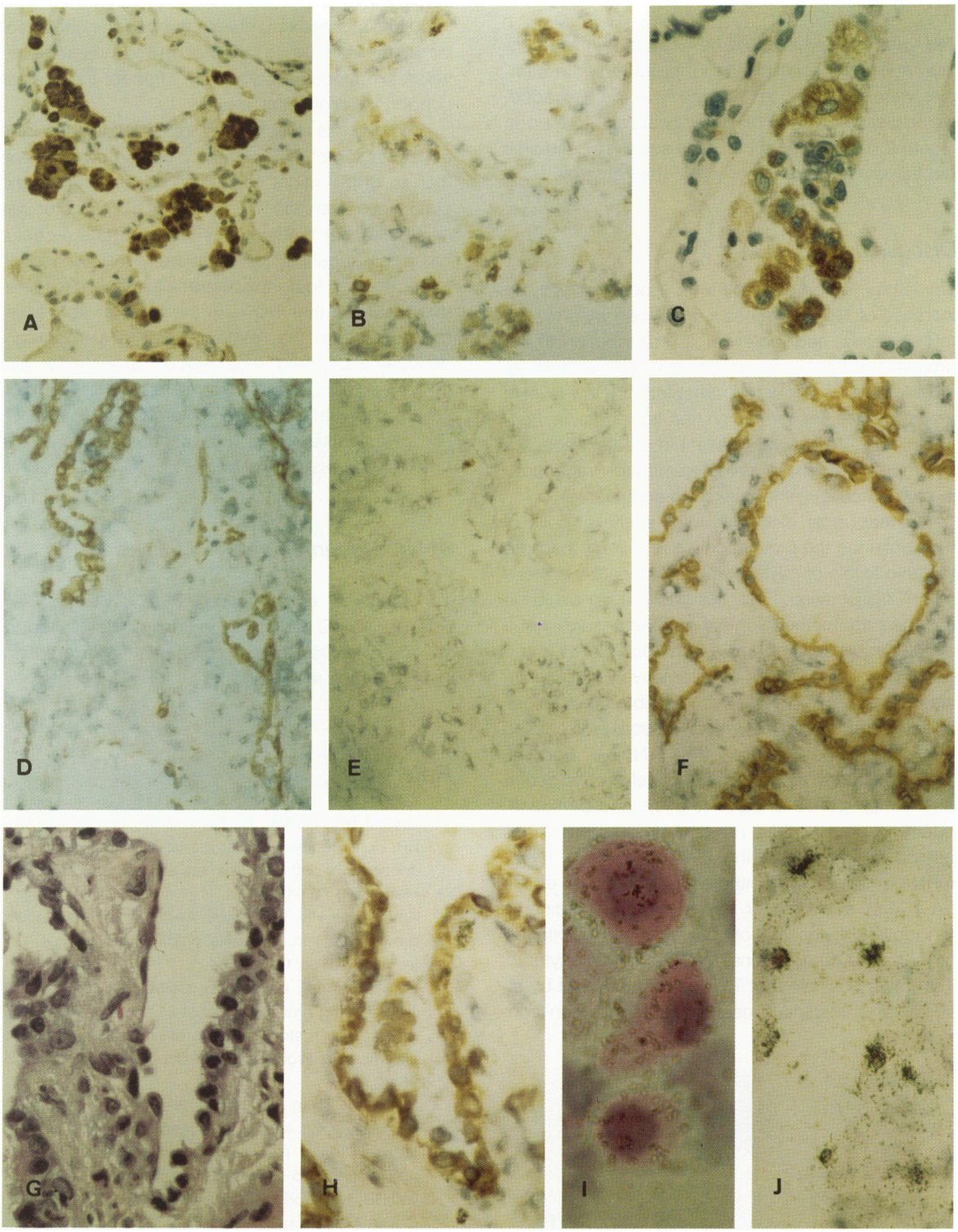

Figure 1. **A** *normal lung: macrophages are identified by the anti-CD68 MoAb.* **B** *normal lung: localization of TNF-α in intra-alveolar cells.* **C** *normal lung: higher magnification of the staining with anti-TNF.* **D** *IPF: staining of epithelial lining (presumably type II cells) with anti-TNF antibody.* **E** *IPF: control staining with nonimmune mouse IgG.* **F** *IPF: staining of alveolar epithelial cells with anti-keratin MoAb.* **G** *IPF: HE staining showing the thickened alveolar septae partially lined by cuboidal epithelial cells.* **H** *IPF: after serail section to* **G**, *stained with anti-TNF MoAb.* **I** *IPF: in situ hybridization with a TNF-α probe showing grains over intra-alveolar cells.* **J** *IPF: in situ hybridization with a TNF-α probe, showing grains over interstitial cells and some of the cells lining the alveolar wall. A and B; 250×, C, G, H, J; 640×, D, E, F; 400×, I; 1600×.*

TNF-α mRNA levels

TNF-α mRNA levels could be evaluated in two controls and two cases of IPF. In the others, the samples were either too small or the RNA too degraded, as assessed by GAPDH hybridization. As seen in Figure 2, TNF-α mRNA could be seen in all samples but the levels were higher in IPF than controls.

Discussion

This study illustrates that IPF is associated with an increase of TNF-α synthesis, as shown by elevated mRNA levels, and moreover a redistribution of the protein within the lung. Indeed, TNF-α and its mRNA were, as expected, detected at a low level in macrophages in normal or fibrotic lungs: in contrast, in IPF, TNF-α was present in greatest concentration in the epithelial cells (presumably regenerating type II cells) lining thickened septae.

Whereas most of the alveolar epithelial cells of IPF contained the protein, only a few, if any, had detectable levels of mRNA, which suggests a protein/mRNA dissociation. First, this observation could be ascribed to methodological problem, such as a cross-reaction of anti–TNF-α MoAbs with an aberrant epithelial epitope, or alternatively, an insufficient sensitivity of *in situ* hybridization; these possibilities appear unlikely for reasons discussed previously (see Results, IPF; TNF-α). Second, TNF-α might be produced by epithelial cells during a restricted period of their life cycle and be subsequently stored in their progeny. In support of this are observations showing the presence of TNF-α mRNA in some lung epithelial cell lines such as the LA-4 lung adenoma or the A549 lung carcinoma (unpublished observation), as well as in epithelial tissues from other origins, such as keratinocytes.[10] Third, it is possible that TNF-α is synthesized by alveolar and interstitial macrophages and then concentrated by the modified alveolar epithelium. This hypothesis is consonant with the capacity of alveolar epithelial cells to absorb and concentrate diverse exogenous substances.[11] Further studies are needed to resolve this issues.

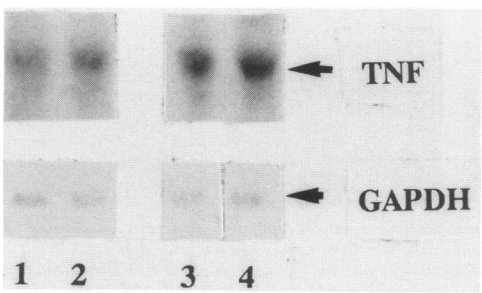

Figure 2. *TNF-α mRNA detected in Northern blots of lung RNA.* **Lanes 1** *and* **2**, *normal lungs;* **lanes 3** *and* **4**; *IPF no. 1 and 4 from Table (4 μg/lane). The position of the TNF-α and GAPDH mRNA are indicated by an arrow.*

Whatever its mechanism of accumulation, the concentration of TNF-α in the modified alveolar epithelium of IPF might impact on the epithelium and continuous interstitium. The toxicity of TNF-α for various cells after binding with its membrane receptor has been frequently demonstrated; TNF-α has also been reported to be toxic intracellularly.[12] It is therefore plausible that TNF-α in the alveolar epithelium contributes to the ongoing epithelial damage, desquamation, and regeneration associated with IPF. In addition, its possible release within the interstitium might induce the proliferation and modulation of interstitial cells, thus contributing to the fibrosing process central to IPF.

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